**SUPPORTING INFORMATION**

# Functionalized α-Helical Peptide Hydrogels for Neural Tissue Engineering

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## Additional Figures and Videos

**Number of Pages: 10**

**Number of Figures: 5**

**Number of Videos: 8**

A

| Peptide name | Sequence |
|---|---|
| Heptad repeat | gabcdef gabcdef gabcdef gabcdef |
| $hSAF_{AAA-W}$ p1 | KIAALKA KIAALKA EIAALEW ENAALEA |
| $hSAF_{AAA-W}$ p2 | KIAALKA KNAALKA EIAALEW EIAALEA |
| $hSAF_{AAA-W}$ p1$N_3$ | zIAALKA KIAALKA EIAALEW ENAALEA |
| RGDS | Prop-GSGYGRGDS |

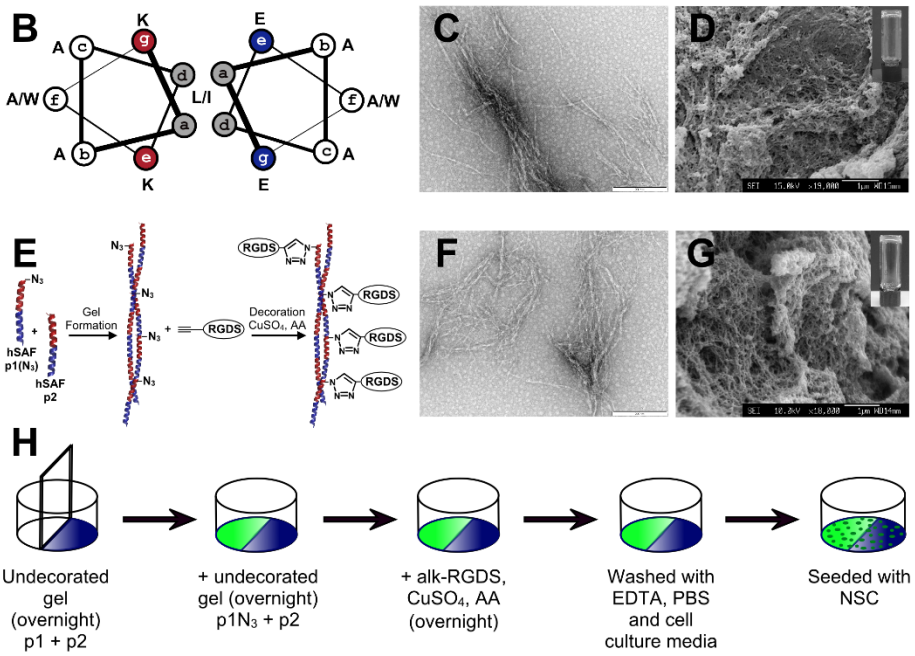


**Figure S1** Peptide sequences, the assembly of undecorated hSAF and RGDS-decorated hSAF gels and the half-moon model. (A) Peptide sequences used for this study. (B) Helical wheel showing the design and interactions of hSAF-p1 and hSAF-p2 (adapted from[1]). (C) By combining hSAF-p1 and hSAF-p2 undecorated fibres with a diameter of 13 ± 5 nm were produced. (D) At 1 mM concentration of hSAF-p1 and hSAF-p2 porous undecorated gels were formed. These gels were clear and self-supporting (inset). (E) To produce RGDS-decorated fibres hSAF-p1 was *N*-terminally azido modified before combining with hSAF-p2. RGDS was then added in the presence of $CuSO_4$ with ascorbic acid (AA). (F) The modification and decoration of hSAFs produced fibres with a similar diameter to undecorated fibres (17 ± 4 nm). (G) RGDS-decorated gels were also porous, clear and self-supporting (inset) at 1 mM of each peptide. (H) Half-moon gels, with half undecorated and half RGDS-decorated semi-circular sides, were formed in 24-well cell culture plates for the migration studies. Scale bars: 200 nm (C and F); and 1 μm (D and G). Key: z, azido norleucine; Prop, propiolate. Undecorated hSAF gel, blue; and RGDS-decorated hSAF gel, green. Figure adapted from[2].

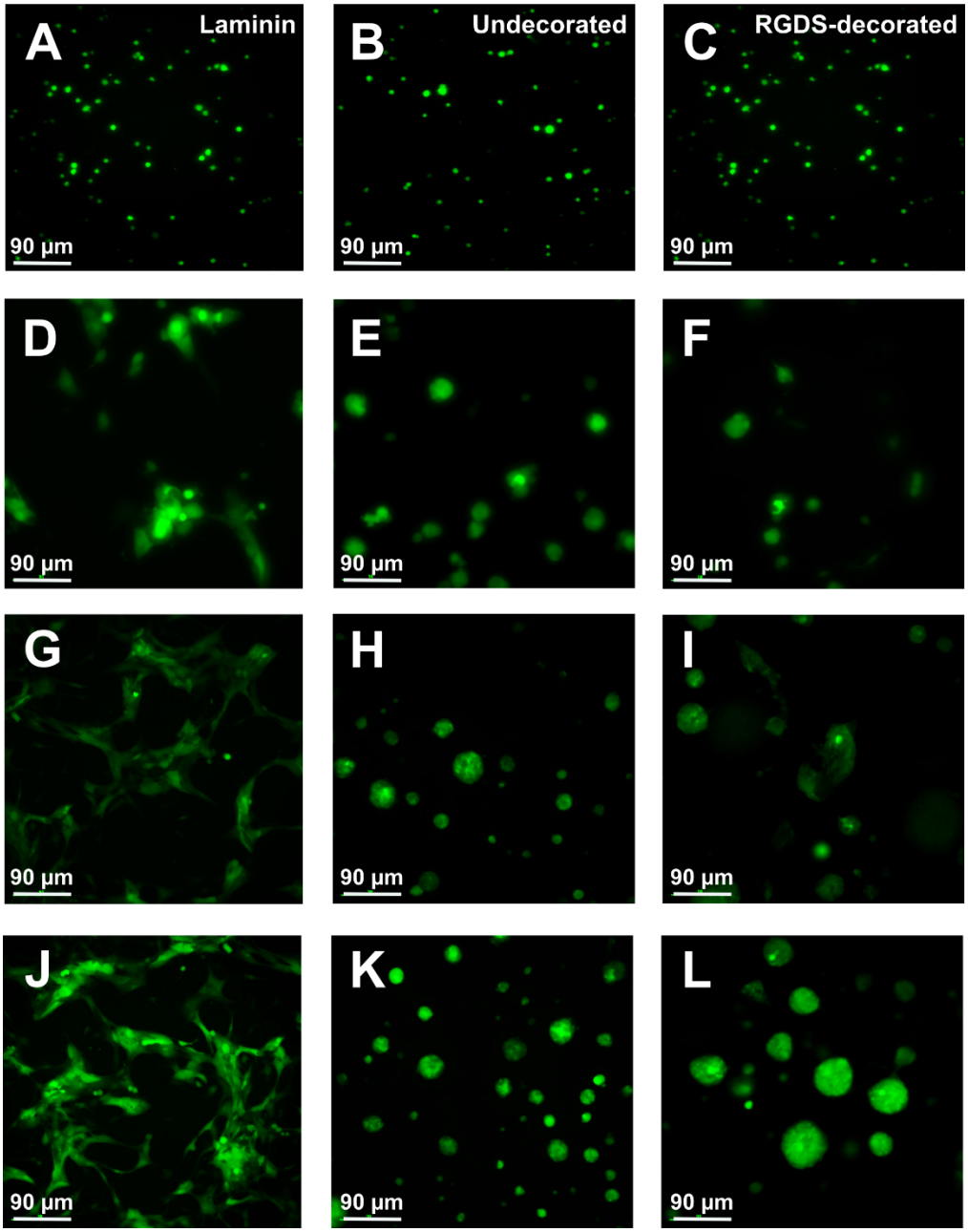


**Figure S2** Morphology and proliferation of neural stem cells over 14 days. GFP-nestin-tagged neural stem cells were seeded onto the gels and imaged using confocal microscopy on day 0 (A-C), day 3 (D-F), day 7 (G-I) and day 14 (J-L). The formation of neurospheres in hSAF gels, but not laminin, was seen by day 3 of the study. Key: GFP-nestin expression, green.

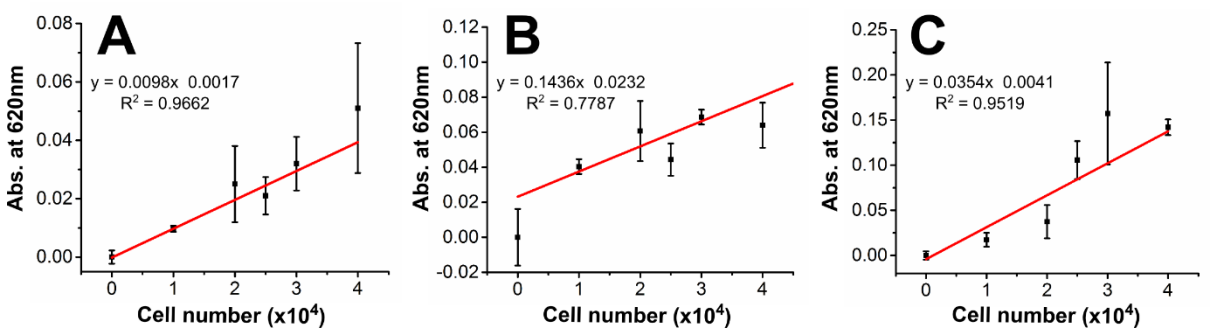


**Figure S3** Calibration curves for assessing metabolic activity of neural stem cells in gels. MTT was used to assess the metabolic activity of neural stem cells on laminin (A), undecorated hSAF gels (B), and RGDS-decorated hSAF gels (C). Cell number and proliferative activity of the cells was then calculated from the calibration curves ($n$ = 3).

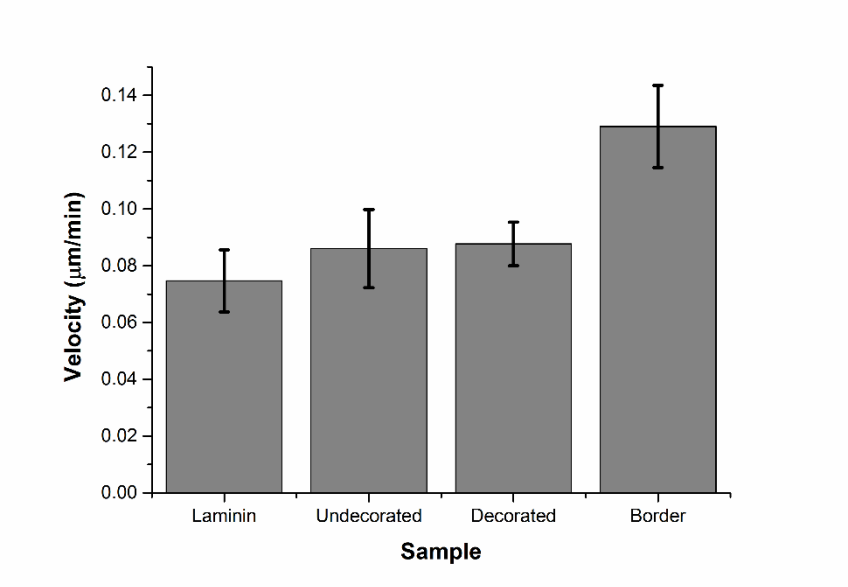


**Figure S4** Velocity of cells migrating across gels. The speed at which cells migrated across the gels was analyzed by tracking their movement over 24 h. While no difference was seen between laminin, undecorated hSAF gel and RGDS-decorated gels, at the half-moon border (between the undecorated and RGDS-decorated hSAF gels) the cells travelled at a greater speed ($p<0.05$). $n$ = 15 (laminin), 24 (undecorated hSAF), 48 (RGDS-decorated hSAF) and 12 (border between undecorated and RGDS-decorated gels).

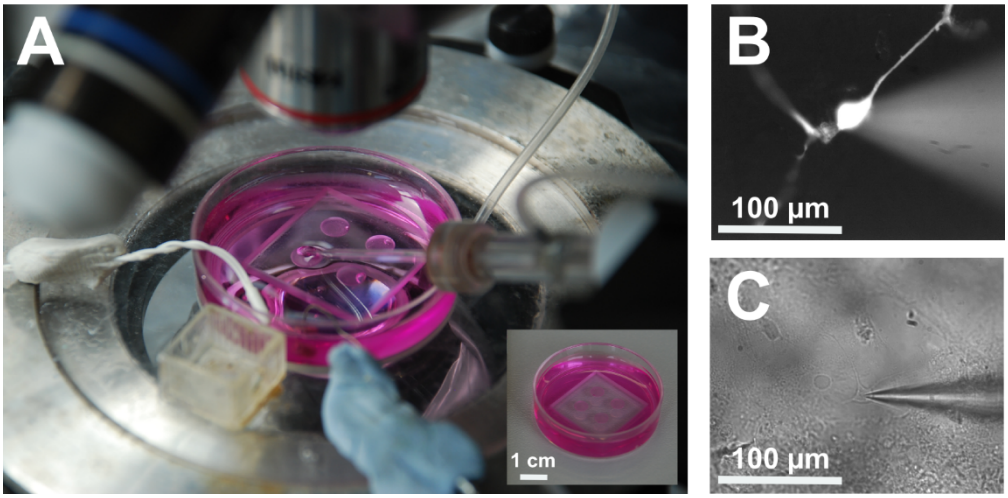


**Figure S5** Electrophysiology set-up. (A) A glass chamber was purpose-built to accommodate 4 gels in 1mm deep wells (2 mm diameter). These gels were then seeded with cells and supported by a 35mm petri dish (inset) allowing the micropipette to reach the cells easily. (B) An infrared differential interference contrast upright microscope with epi-fluorescence ($\lambda_{exc}$ 450 – 490 nm) was used to identify the GFP-nestin tagged cells. (C) Bright-field microscopy was then used to distinguish the cell surface in order to lower the micropipette for patching. $n$ = 12 (undecorated hSAF) and 14 (RGDS-decorated hSAF) for day 7 and 5 (undecorated hSAF) and 10 (RGDS-decorated hSAF) for day 14.

**Video S1**
Depth of NSC penetration into laminin gel. Z-stack confocal images of NSCs cultured in laminin were taken every 0.5 µm for the 14-day cell cultures. The stacks were combined to show NSCs penetration depth into the gel (9 µm). Key: DAPI-stained cell nuclei, blue; and GFP-nestin expression, green.
(MP4)

**Video S2**
Neurosphere formation and depth of NSC penetration into undecorated hSAF gel. Z-stack confocal images of NSCs cultured in undecorated hSAF gel were taken every 0.5 µm for the 14-day cell cultures. The stacks were combined to show NSCs penetration depth into the gel (21 µm). Key: DAPI-stained cell nuclei, blue; and GFP-nestin expression, green.
(MP4)

**Video S3**
Neurosphere formation and depth of NSC penetration into RGDS-decorated hSAF gel. Z-stack confocal images of NSCs cultured in RGDS-decorated hSAF gel were taken every 0.5 µm for the 14-day cell cultures. The stacks were combined to show NSCs penetration depth into the gel (30 µm). Key: DAPI-stained cell nuclei, blue; and GFP-nestin expression, green.
(MP4)

**Video S4**
Formation of connections between neurospheres in RGDS-decorated hSAF gel. Z-stack confocal images of NSCs cultured in RGDS-decorated hSAF gel were taken every 0.5 µm for

the 14-day cell cultures. The stacks were combined to show the formation of connections between neurospheres in the gel (cell penetration depth = 26 μm). Key: DAPI-stained cell nuclei, blue; and GFP-nestin expression, green.
(MP4)

**Video S5**
Migration and spider plots of NSCs over laminin. Left: Phase-contrast images of NSCs taken every 10 min over 24 h on laminin gels were combined to show the migration of cells over time. A representative video is shown. The red line indicates manual tracking of each cell's movement between frames, while blue arrowheads show the direction of cell movement. Right: Spider plots showing the migration tracks of NSCs on laminin over 24 h. The center of the plot denotes the start position of each cell and the dot denotes the end position. Each line represents a single cell. Tracks indicate that the movement of the cells is random, *i.e.* no specific directionality ($n$ = 15).
(MP4)

**Video S6**
Migration and spider plots of NSCs over undecorated hSAF gel. Left: Phase-contrast images of NSCs taken every 10 min over 24 h on undecorated hSAF gels were combined to show the migration of cells over time. A representative video is shown. The red line indicates manual tracking of cell movement between frames, while blue arrowheads show the direction of movement. Right: Spider plots show the migration tracks of NSCs on laminin over 24 h. The center of the plot denotes the start position of each cell and the dot denotes the end position. Each line represents a single cell. Tracks indicate that the movement of the cells is random and in no specific direction ($n$ = 24).
(MP4)

**Video S7**
Migration and spider plots of NSCs over RGDS-decorated hSAF gel. Left: Phase-contrast images of NSCs taken every 10 min over 24 h on RGDS-decorated hSAF gels were combined to show the migration of cells over time. A representative video is shown. The red line indicates manual tracking of cell movement between frames, while blue arrowheads show the direction of movement. Right: Spider plots show the migration tracks of NSCs on laminin over 24 h. The center of the plot denotes the start position of each cell and the dot denotes the end position. Each line represents a single cell. Tracks indicate that the movement of the cells is random and in no specific direction ($n$ = 48).
(MP4)

**Video S8**
Migration and spider plots of NSCs across the hSAF half-moon border. Left: Phase-contrast images of NSCs taken every 10 min over 24 h across half-moon hSAF gels were combined to show the migration of cells over time. A representative video is shown where the left side represents the RGDS-decorated gel and the right side represents the undecorated gel. The red line indicates manual tracking of cell movement between frames, while blue arrowheads show the direction of movement. Right: Spider plots show the migration tracks of NSCs on laminin over 24 h. The center of the plot denotes the start position of each cell and the dot denotes the end position. Each line represents a single cell. Tracks indicate that the cells move towards the RGDS-decorated gel ($n$ = 12).
(MP4)

## Materials and Methods

**Peptide Synthesis.** hSAF-p1, hSAF-p1($N_3$) and hSAF-p2 (Figure S1) were synthesized on a H-Ala-HMPB-ChemMatrix resin, whilst RGDS was synthesized on a Rink Amide-ChemMatrix resin (PCAS BioMatrix Inc., Canada). All syntheses were done on a 0.5 mmol scale using `Liberty' microwave-assisted peptide synthesizers (CEM, UK) and standard 9-fluorenyl-methoxycarbonyl (FMOC)-based solid-phase chemistry. α-Fmoc-ε-azido-norleucine was manually coupled using 2 eq. amino acid, 1.9 eq. O-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate and 2.5 eq. *N,N*-diisopropylethylamine. For the alkyne modification of the RGDS peptide 5 eq. propiolic acid, 6 eq. hydroxybenzotriazole and 4.5 eq. *N,N′*-diisopropylcarbodiimide were used. Cleavage of the peptides with complete removal of side-chain protecting groups was achieved by incubation with a trifluoroacetic acid-based (TFA) mixture (TFA:triisopropylsilane:water, 95:2.5:2.5) for 3 h. Cleaved products were precipitated in cold diethyl ether, centrifuged, the precipitates were dissolved in 5 mL of a 1:1 mixture of acetonitrile/water, and then freeze-dried to give fine white solids.

**Peptide Purification**. All peptides were purified by reverse-phase high-performance liquid chromatography (HPLC; JASCO, UK) using a Vydac® TP C18 column (10 μm, 22 × 250 mm) under acidic conditions (buffer A: 0.1% TFA in $H_2O$; buffer B: 0.1% TFA in acetonitrile). Depending on the peptide sequence, different gradients were used: hSAF-p1, hSAF-p1($N_3$) and hSAF-p2 were purified using a 20 to 60% buffer B gradient over 60 min; alk-RGDS was purified using a 5 to 40% buffer B gradient over 60 min. The purified peptides were freeze dried and stored at 4 ˚C in the dark.

**Peptide Characterization.** Peptide masses were confirmed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) on an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF instrument (matrix: α-cyano-4-hydroxycinnamic acid (CHCA), external calibration). All peptides were of >95% purity as monitored by analytical HPLC (JASCO, UK) using a Phenomenex Prodigy ODS-3 column (5 μm, 4.6 × 100 mm). Peptide concentrations were determined by UV absorbance ($\varepsilon_{280}$(Trp) = 5690 $mol^{-1}$ $cm^{-1}$, $\varepsilon_{280}$(Tyr) = 1280 $mol^{-1}$ $cm^{-1}$) using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK).

**hSAF Scaffold Formation**. 2 mM stock solutions of the complementary peptides (hSAF-p1 or hSAF-p1($N_3$), plus hSAF-p2) in 3-(*N*-morpholino) propanesulfonic acid (MOPS)-buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA) pH 7.4 were mixed, to give a final concentration of 1 mM of each peptide. The peptide mixture was set on ice for 10 min, followed by overnight incubation at 4 °C. For undecorated hSAF gels hSAF-p1 and hSAF-p2 were used, whereas for RGDS-decorated hSAF gels hSAF-p1($N_3$) and hSAF-p2 were used. Decoration was achieved by pre-mixing 4 mM $CuSO_4$, 4 mM ascorbic acid and 2 mM of alk-RGDS, followed by addition to and incubation with the azide-containing gel overnight at 4 °C. The $CuSO_4$ catalyst was then removed by washing the gel three times with 10 mM EDTA, three times with phosphate buffered saline (PBS; 160 mM NaCl, 3 mM KCl, 8 mM $NaHPO_4$, 1 mM $KH_2PO_4$, pH 7.4) for ½ h each wash, and then perfused with NSC media before the cell-culture experiments.

Half-moon gels were prepared as outlined in Figure S1.[1] Briefly, sterile glass coverslips were vertically mounted as separators in 24-well tissue-culture plates (CellStar, Greiner Bio-one Ltd, UK). One half of the well was filled with the undecorated hSAF gel solution and incubated overnight at 37 °C. After removal of the coverslip the second half of the well was filled with the hSAF-p1($N_3$) and hSAF-p2 peptide mix. This was again left overnight at 4 °C and decorated the following morning with pre-mixed 4 mM $CuSO_4$, 4 mM ascorbic acid and 2 mM of alk-RGDS. After further incubation overnight at 4 °C, the $CuSO_4$ was removed using the previously described protocol before media perfusion and cell seeding.

**Laminin Scaffold Formation.** 96-well tissue-culture plates were coated with 50 μL poly-D-lysine (50 μg/ mL in PBS) for 1 h. After removal of the poly-D-lysine solution the plates were washed three

with PBS. 50 µL laminin solution (20 µg/ mL in PBS) was added to each well and incubated at 37 °C for 30 min. The plates were washed twice with PBS before use.

**Isolation of Murine Embryonic Neural Stem Cells (NSCs).** Embryonic E12 – E14 C57/Black6 murine cortices were dissected into ice-cold DMEM/F-12 media containing penicillin – streptomcyin (dissection solution; 100 U/ mL in PBS). The meninges were further isolated in a sterile 35 mm tissue-culture dishes containing fresh dissection solution. The cortex tissue was digested in 500 µL accutase at 37 °C for 10 min. The enzyme was inactivated by adding 500 µL NSC media (1:1 DMEM/F-12, 1% v/v MEM non-essential amino acids (100X), 2% v/v B-27 supplement, 1% v/v 100X penicillin – streptomycin, 10 ng/mL recombinant human epidermal growth factor and 10 ng/mL basic recombinant human fibroblast growth factor). The isolated cells were centrifuged at 1200 rpm for 2 min, the supernatant removed and the cells resuspended in 1 mL NSC media. Single-cell suspensions were achieved through mechanical agitation. Isolated cells were incubated in fresh NSC media at 37 °C, 5% $CO_2$. GFP-positive cells were produced according to a previously reported protocol.[3] Briefly, a nestin enhancer EGFP construct was used to transfect the primary NSCs. These cells were then selected with G418 (200 µg/mL) in NSC media for 4 weeks. GFP-positive cells were isolated to establish the nestin-GFP NSCs used in this study. The cells were incubated in fresh NSC media at 37 °C, 5% $CO_2$.

**NSC Culture.** Neurospheres were centrifuged at 1500 rpm for 5 min and digested into single-cell suspensions using accutase (Sigma-Aldrich, UK) for 1 - 2 min at 37 °C. The cells were resuspended in fresh NSC media and passed through a 40 µm cell strainer to remove remaining neurospheres. The single-cell suspension was incubated at 37 °C, 5% $CO_2$. Media was replaced every 3 days and the cells passaged every 6 days.

**NSC Seeding.** Accutase-digested cells suspended in NSC media were counted in the presence of Trypan Blue (Sigma-Aldrich, UK) using a hemocytometer. Gels were made as previously outlined in 96-well or 24-well tissue-culture plates. Gels in 96-well plates were seeded with $0.5 \times 10^4$ to a total volume of 200 µL NSC media whilst those in 24-wells were seeded with $1.0 \times 10^4$ to a total volume of 500 µL. The seeded samples were incubated at 37 °C, 5% $CO_2$ and 100% relative humidity (Galaxy S+, RS Biotech, Eppendorf UK Limited, UK) and the media changed every 3 days. For differentiation the media used did not contain either of the afore-mentioned growth factors. All other conditions were kept the same.

**Metabolic Activity Assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).** The proliferation rate of NSCs on hSAF and laminin gels was evaluated by assessing cell metabolic activity on 0, 3, 7 and 14 days post-seeding. Briefly, 10 µL MTT (10% v/v tetrazolium MTT, Sigma-Aldrich, UK, in PBS) was added to cell-seeded scaffolds with NSC media. After 18 h incubation at 37 °C, 200 µL HCl-isopropanol was added to the well and incubated for a further 45 min at 37 °C. The absorbance of the resulting solution was measured in triplicate, at 620 nm (SpectraMax M2, Molecular Devices, USA) and used to calculate the number of metabolizing cells present in each sample (Figure S3; protocol adapted from).[4]

**NSC Migration.** NSCs were seeded on half-moon gels and time-lapse images recorded every 10 min for 24 h using Deltavision imaging system with a motorized XYZ-stage (Applied Precision GE Healthcare, USA). Cell migration was quantified as described previously.[5] Briefly, cell nuclei were traced and their X/Y co-ordinates at each time point, relative to their start position, exported to Microsoft Excel. Cellular displacement (Td) was calculated from the co-ordinates recorded for each cell at the end of the 24 h observational period using Pythagoras' theorem: $Td = \sqrt{((X_{end} - X_{start})^2 + (Y_{end} - Y_{start})^2)}$. A mean value was then obtained from all cells on that region of the gel. To obtain the direction of migration, the angle each cell moved with respect to its starting position (θ) was calculated trigonometrically: $\theta = \cos^{-1} (X_{end} - X_{start})/ Td$. The cosine of the angle was then calculated for each cell to give a value of between -1 and +1. A value of -1 depicted a horizontal trajectory directly to the left (RGDS-decorated side), whereas +1 represented a cell moving in the opposite

direction, *i.e*. to undecorated side. More-vertical directions of migration gave values closer to 0. Averaging the cosines for every cell on a region of the gel, yielded average directedness of cell movement. Variation in the number of cells tracked for migration was due to the limited number of observable cells that fit experimental criteria. Only single cells were included in the study. Similarly, cells that proliferated or moved out of view were ignored. Finally, there were practical limitations with sample size which reduced the area where the frames were set up.

**Immunocytochemistry for Protein Expression and Fluorescent Microscopy.** After removing the NSC media the samples were washed twice with PBS. The cells were fixed with 4% v/v paraformaldehyde (Sigma-Aldrich, UK) for 20 min and washed twice again with PBS. The cell membranes were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min at 22 °C. The samples were then pre-incubated with bovine serum albumin (5% v/v in PBS) at 22 °C for 30 min to block non-specific binding. 100 µL primary antibodies (rabbit polyclonal anti-MAP2 and mouse monoclonal anti-GFAP 1:200 in PBS, Cell Signaling Technology, UK) were diluted with 100 µL 0.1% Triton X100 in PBS (PBS-Tx) and left at 4 °C overnight. The following day the primary antibodies were removed, the samples washed with PBS-Tx three times for 5 min each. 100 µL of the TRITC-conjugated (Alexa Fluor 594) goat anti-mouse and anti-rabbit secondary antibody IgG was added (1:500 in PBS). The sample was covered with foil to prevent photobleaching and left at 22 °C for 1h. The secondary antibody was then removed and the samples washed with PBS-Tx four times for 5 min each. The samples were then mounted with VECTASHIELD mounting medium containing DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories, UK), sealed with a coverslip and clear nail varnish and observed using MetaMorph software (Universal Imaging Corporation) on a Delta-vision system with a motorized XYZ-stage using either a fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate filter. Z-stacks were collated on a Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope (Leica Microsystems Ltd, UK). The number of processes produced by each cell were counted using ImageJ software.

**Electrophysiology.** A patch-clamp setup designed for brain-slice recordings was adapted for this study. Specifically, a glass-recording chamber was created into which hSAF gels were directly assembled (Figure S5), and seeded with NSCs at a density of 5000 cells per well. The customized chamber was then transferred to a standard petri dish containing NSC medium and incubated at 37 °C, 5% $CO_2$ and 100% relative humidity. The medium was replaced every 3 days with 3 mL of differentiation medium.

Electrophysiological recordings were performed after 7 and 14 days culture in differentiation medium. Petri dishes containing the customized chamber were transferred to the microscope and the culture media was removed. The NSCs were continuously perfused with Hank's Balanced Salt Solution containing 130 mM NaCl, 3 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-free acid, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM glucose, pH 7.3, 32 °C. Cells were visualized using infrared differential interference contrast microscopy combined with epi-fluorescence (Olympus, Japan). Standard glass micropipettes of resistance 2.5 – 6 MΩ were used for recordings. The micropipettes were filled with an internal solution suitable for measuring voltage-gated $K^+$ currents (135 mM K-gluconate, 5 mM NaCl, 10 mM K-HEPES, 200 µM EGTA, 300 µM Na-GTP, 4 mM Mg-ATP, 13.4 mM biocytin, pH 7.3). The liquid junction potential that arose from the combination of bath and pipette solution was corrected arithmetically.

All measurements were made using a Multiclamp 700B patch clamp amplifier (Molecular Devices, USA). Data were low-pass filtered (5 – 10 kHz), digitized (100 kHz) and subsequently visualized and stored on a PC using pClamp electrophysiology software. Once a whole-cell-recording configuration was established, the pipette capacitance was neutralized and the series resistance compensated (10% – 80% correction). Voltage-clamp recordings were made for the quantitative evaluation of voltage-gated currents.

**Statistical Analysis.** Data are presented in the format "mean ± standard error of the mean (SEM)". Differences in mean values were compared within groups and significant differences were determined by ANOVA (Analysis Of Variance) with post-hoc Tukey-Kramer HSD (honestly significant difference) test. The significance level was set at $p< 0.05$.

For the electrophysiology experiments specifically, the peak ratio of the averaged trace evoked by a +70 mV voltage-step was compared between gels and over time. Similarly, the maximal conductance values and the values for half the voltage that generated half the maximal conductance for $K^+$ current activation were compared between groups over time with two-way ANOVA. If an overall effect of gel, time or gel vs time was observed, a Bonferroni post-hoc t-test was performed. The same statistical approach was used to compare the membrane passive properties between gels over time. The time of rise between 10% and 90% of the outward current peak (10 – 90 time of rise) at 14 days, was compared between gels with an unpaired Student's t-test.

## References


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